DELIVERY SYSTEM FOR PORCINE SOMATOTROPIN

Field of the Invention:

The present invention relates to an expression construct for delivering an exogenous polypeptide to a host. The present invention also relates to recombinant cells which include this expression construct and to semi-permeable capsules which include the recombinant cells.

Background of the Invention:

In mammals, somatotropin (growth hormone) is normally secreted from the pituitary gland. However, exogenous administration of somatotropin to pigs has been shown to improve feed efficiency 15-20%, increase daily weight gain 10-15%, reduce carcass fat 10-20%, increase lean meat content 5-10% and reduce feed intake. Unfortunately, somatotropin (which is a small protein of 190 amino acids) is susceptible to gastric acids and protein digestion hence daily injections are required in order to be efficacious. Currently, welfare and ethical issues discourage the use of the pneumatic pST injection gun and the costs of daily administration restrict industry-wide adoption.

Recent advances in gene therapy have enabled the development of strategies which avoid the dependence on autologous target cells and immunosuppressive therapy by utilising transfected cells encapsulated in a semi-permeable alginate-poly-L-lysine-alginate (APA) membrane. The APA capsule environment is compatible with cell viability and growth so that transfected cells remain viable, secreting growth factors, for extended periods. The APA is permeable to small proteins and consequently gene expression can be controlled by external means. The APA barrier inhibits immune surveillance and cell rejection events so that non-host, highly expressing, cells can be employed in the capsule. The APA barrier may also prevent uncontrolled proliferation of the transfected cells in the recipient host. The APA capsule can be removed, potentially re-used, in order to negate the concerns regarding consumption of transgenic material. Further, if the capsule is damaged by severe tissue trauma a normal host-graft rejection would destroy the implanted cells.

Summary of the Invention:

The present inventors have now found that ligation of an insulin secretory signal to a heterologous gene sequence prior to introduction of the gene sequence into a host cell results in a surprising increase in the level of secretion of the heterologous gene product. This finding has led to the development of an improved gene delivery system involving encapsulation of recombinant cells for implantation into a host.

Accordingly, in a first aspect, the present invention provides an expression cassette including a sequence encoding an insulin secretory signal operably linked to a heterologous sequence encoding a polypeptide.

By "heterologous sequence" we mean a sequence other than a sequence encoding insulin.

By "operably linked" we mean that the insulin secretory signal sequence is contiguous and in reading frame with the heterologous coding sequence.

The preferred insulin secretory signal is an insulin secretory signal having the amino acid sequence shown as SEQ ID NO:1. However, it will be appreciated by those skilled in the art that a number of modifications may be made to that secretory signal without deleteriously affecting the biological activity of the signal. For example, this may be achieved by various changes, such as sulfation, phosphorylation, nitration and halogenation; or by amino acid insertions, deletions and substitutions, either conservative or non-conservative (eg. D-amino acids, desamino acids) in the peptide sequence where such changes do not deleteriously affect the overall biological activity of the secretory signal. Thus, the inclusion in the expression cassette of an insulin secretory signal which has been modified in one or more of the abovementioned ways, is to be regarded as being encompassed by the present invention.

The heterologous sequence may encode any polypeptide, other than insulin, of interest. For example, the heterologous sequence may encode a hormone, cytokine, receptor agonist or antagonist, pheromone or enzyme. In a preferred embodiment, the heterologous sequence encodes a growth hormone. Preferably, the growth hormone is somatotropin.

In a second aspect, the present invention provides a vector including an expression cassette of the first aspect. The vector may be any suitable

vector for introducing the expression cassette into a cell. Suitable vectors include viral vectors and bacterial plasmids.

The expression cassette of the first aspect of the present invention, or the vector of the second aspect, may further include one or more elements which regulate gene expression. Examples of suitable regulatory elements include the Melatonin Response Element (MRE) (as described in Schrader *et al, 1996*, the entire contents of which are incorporated herein by reference), and/or rapamycin mediated transcription factors (as described in Magari *et al, 1997*, the entire contents of which are incorporated herein by reference). In a preferred embodiment, the regulatory element(s) enable pulsatile expression of the polypeptide of interest.

In a third aspect, the present invention provides a recombinant cell which includes an expression cassette according to the first aspect of the present invention.

The recombinant cell may be a bacterial, yeast, insect or mammalian cell. In a preferred embodiment, the recombinant cell is a mammalian cell. In a further preferred embodiment, the cell is a rat myoblast (L6) cell.

In a fourth aspect, the present invention provides a method of producing a polypeptide which includes culturing a recombinant cell of the third aspect under conditions enabling the expression and secretion of the polypeptide and optionally isolating the polypeptide.

The recombinant cell(s) of the present invention may be encapsulated in a semi-permeable matrix for delivery or implantation in a host.

Accordingly, in a fifth aspect, the present invention provides a capsule for implantation in a host, the capsule including a semi-permeable membrane which encapsulates one or more recombinant cells according to the third aspect of the present invention.

In a preferred embodiment, the semi-permeable membrane is an alginate-poly-L-lysine-alginate (APA) membrane. The preparation of an APA semi-permeable membrane is described in Basic *et al, 1996*, the entire contents of which are incorporated herein by reference.

In a sixth aspect, the present invention provides a method of administering a polypeptide to a host which includes administering to the host an expression cassette according to the first aspect of the present invention.

In a seventh aspect, the present invention provides a method of administering a polypeptide to a host which includes implanting in the host a capsule according to the fifth aspect of the present invention.

The host may be any animal or human. In a preferred embodiment, the host is a livestock animal. In a further preferred embodiment, the host is selected from the group consisting of grazing cattle, feed-lot cattle, dairy cows, pigs and poultry.

It will be appreciated by those skilled in the art that the present invention provides an improved system for the delivery of genetic material to a host. The ligation of the insulin secretory signal to a biologically active polypeptide leads to increased secretion of the polypeptide from recombinant cells. Following secretion, the secretory signal may be cleaved leaving the biologically active polypeptide. The recombinant cells, when encapsulated in a semi-permeable membrane, have the capacity to secrete significant amounts of the biologically active polypeptide and the semi-permeable membrane enables control of gene expression by external means. Implantation of the encapsulated recombinant cells provides an advantage in that the implantation requires minimal surgery. Further, the semi-permeable membrane reduces immune surveillance and cell rejection which means that non-host cells can be employed in the capsule.

In a preferred embodiment, the semi-permeable membrane is durable which provides an advantage in that it may limit cell growth thereby preventing uncontrolled proliferation in the recipient host. The capsules provide a further advantage in that they may be removed and re-used.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting Examples and Figures.

Brief description of the accompanying figures:

~~**Figure 1:** Insulin secretory signal - pST gene construct.~~

~~**Figure 2:** Insulin secretory signal - pST peptide sequence.~~

Figure 3: Rate of weight gain (from day 0) for control and individual pST-L6IXS treated pigs.

Figure 4: Percentage weight gain for control and individual pST-L6IXS treated animals.

Figure 5: Plasma, pST levels for control and individual pST-L6IXS treated animals.

Figure 6: Plate 1- Appraisal of pST-L6IXS capsule administration site
Plate 2 - Placement of pST-L6IXS capsule in culture media for ex-vivo assessment.

Figure 7: Ex-vivo assessment of secretion of pST from capsules for a 24 hr period following removal from host animal.

Figure 8: Mean plasma pST (over 3 hours @ 30 min intervals) before (white bars) and 1 week post pST capsule administration (black bars) (*significant).

Figure 9: Daily plasma pST concentrations of two pigs, pig 206 and 228, with implanted capsules secreting 25 ng/ml and 500 ng/ml respectively.

Figure 10: Rate of Gain (ROG) in kg/day (black squares) and P2 back fat measurements in pigs produced in Example 4.

Figure 11: Rate of Gain (ROG) of male pigs following implantation with pST secreting or control immunoneutral gene therapy (IGT) capsules (± SEM).

Figure 12: Back fat (P2) of male pigs following implantation with pST secreting or control immunoneutral gene therapy (IGT) capsules (± SEM).

Figure 13: Loin (eye) muscle area of male pigs following implantation with pST secreting or control immunoneutral gene therapy (IGT) capsules (± SEM).

Detailed description of the invention:

Example 1: Cloning of the ISS-pST construct

The pST gene was obtained from Southern Cross Biotechnology Pty Ltd in an *E. coli* bacterium. The plasmid containing the pST gene, pMG939, was isolated from the bacterium using standard plasmid preparation techniques. The PCR primers were designed to amplify the pST gene, add an *Xho* I site to the 5' end and an *Xba* I site to the 3' end to enable ligation events.

The modified pST gene sequence was subsequently ligated to a secretory signal sequence (ISS) derived from the preproinsulin cDNA. *Nhe* I (GCTAGC) and *Xba* I (TCTAGA) restriction sites were constructed in front of the ISS start codon and after the 3' terminal codon of pST, respectively, to allow incorporation into the pCI-neo plasmid (Promega). The pST fusion construct was subsequently isolated and sequenced to verify the coding region (Figure 1).

Transfection of rat myoblast (L6) cells (pST gene incorporation into cells) was performed, with LipoTAXI (Stratagene), 2hrs after the L6 cells were trypsin treated. pST transfected L6 cell clones were maintained in culture, selected with G418, until $>10^7$ cells were generated. Aliquots (2ml) of the culture supernatant were stored at -20°C prior to assessment of pST concentrations in a pST radioimmunoassay (RIA) established by Dr P. Wynn at Sydney University (Camden). The RIA sensitivity was deemed to be >0.4ng/ml with CV's in the order of 12.4%. The polyclonal antisera was raised in guinea pigs with a pST peptide antigen. The RIA results (Table 1) indicate that the pST gene construct produced protein (Figure 2) which is reeognised by polyclonal antisera raised against the native form of pST, purified from porcine pituitary glands. L6 Clones pCI/pst-1..5 were generated from the modified transfection technique as described below.

Modified transfection protocol

Characteristically, L6 cells adhere to culture plates and require detachment with trypsin to passage cells; transfection is routinely performed 24hrs later. This procedure resulted in L6 cell clones (n=10) secreting pST at 6-18 ng/ml. Applying LipoTAXI (Promega) and the ISS/pST plasmid to the L6 cells 2hrs after trypsin treatment increased the secretion rate of pST 10-20 fold (>180ng/ml, n=5 clones). This higher pST secretion rates reduce the number of cells (capsules) required to enhance growth.

TABLE 1: Concentrations (ng/ml) for each clone transfected with ISS-pST.

L6 clone	pST (ng/ml)
pCI/pst-1*	182
pCI/pst-2*	188
pCI/pst-3*	188
pCI/pst-4*	140
pCI/pst-5*	200
pCI/pst-6	17
pCI/pst-7	12
pCI/pst-8	8
pCI/pst-9	9
pCI/pst-10	7
pCI/pst-11	7
pCI/pst-12	10
pCI/pst-13	8
pCI/pst-14	6
pCI/pst-15	18

Example 2: Preparation of the porcine somatotropin-rat myoblast (L6) immunoneutral expression system (pST-L6IXS)

The encapsulation procedure described in Basic *et al, 1996*, was followed with the following modifications.

Encapsulation of cells at room temperature, utilises calcium chloride (or lactate) [100mM] to gel the alginate [1.5% w/v] droplets followed immediately by washing with saline (0.9% NaCl) then resuspending in poly-L-lysine [0.05%] for 5 min. Calcium chloride crosslinking for 10min at 37°C resulted in an alginate matrix that was more compatible with cell viablity.

After the poly-L-lysine coating and saline washes another alginate layer is added. Sodium citrate [55mM] treatment for 4min at room temperature softens the capsule to a consistency that increases the difficulty of further manipulation. Cell viablity is apparently reduced to <35% with 4 min exposure to sodium citrate. Placing the capsules in a cell strainer prior to sodium citrate treatment enabled 1min exposure, at 37°C, improving cell viability to >98%.

Procedural and equipment modifications to the encapsulation protocol improved the efficiency (time and resources) of encapsulation with routine increases in cell viability in the order of 64%.

Example 3: Pilot experiment (1) involving implantation of pST-L6IXS in pigs

Preliminary results obtained with the pST-L6IXS, administered to growing mice, indicate enhanced growth characteristics. In a pilot experiment with male pigs (n=9, mean live weight 61 kg) varying numbers of pST-L6IXS were administered in different sites (3 capsules, i.m. in the neck muscle, 3 capsules s.c. in the neck, 10 capsules s.c. at the base of the ear, 20 capsules i.m. in the neck or 29 capsules i.m. in the neck of individual animals on day 0). Blood samples (10ml) were collected via jugular venipuncture and P2 ultra-sound (us) measurements were recorded at -14, 0, 7, 14, 21, 28 and 36 days post administration. The sites of pST-L6IXS administration were monitored for tissue reaction events throughout the experiment. On day 36 animals were euthanased and carcass analysis (back fat depth, BF(mm); eyemuscle area, EMA(cm); forearm bone length, BONE(cm); heart weight, HEART(gm); spleen weight, SPLEEN(gm) and liver weight, LIVER(gm) were recorded (see Table 2) and pST-L6IXS recovered. Figure 3 represents the rate of gain (from day 0) for control (con, mean$\pm$SE, n=4) and individual values for pST-L6IXS treated pigs. Percentage weight gain, over the pST-L6IXS treatment is presented in Figure 4 with the mean$\pm$SE for control (con) pigs and individual pST-L6IXS treated animals. Plasma pST (ng/ml) was determined by radioimmunoassay (RIA) and presented in Figure 5, with mean$\pm$SE control (con) and individual concentrations for pST-L6IXS treated pigs. At slaughter the site of pST-L6IXS capsule administration was appraised (Figure 6, Plate 1, arrow) prior to removal and placement in culture media for ex-vivo assessment (Figure 6, Plate 2) of 24 hour secretion of pST (Figure 6). No apparent tissue damage or immune reactions were observed either i.m. or s.c. at day 36. However, the capsules placed in the ear (s.c.) appeared to be highly vascularised and were 100% recoverable. The capsules placed in the neck region were <10% recoverable.

The pST-L6IXS remained patent over 36 days *in vivo* and appeared to proliferate within the capsule (Plate 2) which can be removed in order to negate the concerns regarding consumption of transgenic material. Further, if the capsule is damaged (i.e. by severe tissue trauma) a normal host-graft rejection destroys the L6 cells preventing propagation of transfected material. Experiments in mice and pigs have demonstrated that pST-L6IXS are

efficacious in altering plasma pST, enhancing growth characteristics and potentially immune competence of animals.

TABLE 2

pST-L6IXS PILOT EXPERIMENT:
Pigs (male) supplied by Westmill piggery (Young, NSW)
Experiment at EMAI, maximum security piggery.
ACEC Ref No: 98/20

				LIVEWEIGHT (kg)													
				Date													
				###	##	###	##	###	###	9/07/98							
				Day						(slaughter)	CARCASS						
	Pen	Treatment	Animal	-14	0	7	14	21	28	36	P2us (mm)	BF (mm)	EMA (cm)	BONE (cm)	HEART (gm)	SPLEEN (gm)	LIVER (gm)
	A	con	291	24	67	NR	NR	89	95	100	11	9	54.5	24.5	388.6	159.8	1720.2
C	A	con	292	25	61	NR	NR	84	90	90	8	10	54.9	23.7	381.5	103.2	1703.6
C	B	con	294	22	74	NR	NR	94	103	104	12	15	46.5	24.4	391.5	173.2	1636.5
C	B	con	295	22	55	NR	NR	76	84	91	9	7	50.6	20.0	396.6	138.2	1561.8
T	B	3sc neck*	297	23	67	NR	NR	85	90	91	9	12	45.2	23.5	385.3	177.0	1817.7
		*infected capsule site								CvTp<0.05	nsd	nsd	CvTp<0.06	nsd	CvTp<0.05	nsd	nsd

Example 4: Pilot experiment (2) involving implantation of pST-L6IXS in pigs

A second pilot experiment was conducted in order to optimise pST-L6IXS delivery by capsules so as to achieve growth responses similar to the energy repartitioning observed with daily pST injections.

As shown in Example 1, pST secreting cells have been produced with a range of secretion rates (6-200 ng/ml). pST secretion rates in the order of 2-25 ng/ml appear to be the most stable following the imposition of stress (i.e. by bacterial contamination) on the pST secreting cells (data not shown). Accordingly, clones secreting about 5 ng/ml (clone pCI/pst-14) and about 10 ng/ml (pCI/pst-12) were selected for this pilot experiment. Male pigs (n=10, mean live weight 78.1 kg) were administered various numbers of capsules (produced according to the procedure described in Example 2) s.c. at the base of the ear (Table 3).

Pig	Capsule Number	Clone
204	1	a
216	1	b
230	3	a
202	3	b
226	5	a
206	5	b
208	10	a
224	10	b
222	100	a
228	100	b

a = clone pCI/pst- 14 (5 ng/ml)
b = clone pCI/pst-12 (10 ng/ml)

Body weights were recorded at the beginning and the end of the experiment. Animals were held in individual pens (2 m^2) and stabilised to a controlled environment facility (22^{0}C) for 1 week. The animals were offered *ad libitum* water and standard pelleted grower rations (3 kg/day @ 09:00 hrs),

and daily residues were recorded. Catheters were placed in ear veins (evc), and 24 hours later sampling commenced. Control pig (i.e. no pST capsules) blood plasma (10 ml) was collected every 30 min for 3 hours. pST capsules were administered to the ipsilateral ear immediately following serial sampling. Blood (10 ml) was collected via evc (daily @ 11:00 hrs) while catheters remained patent. Treatment (7 days post administration of pST capsules) blood plasma (10 ml) was collected every 30 min for 3 hours. Slaughter and carcass analysis was performed at about 100 kg live weight 21 days later. pST capsules were then recovered from ears and placed in *in vitro* culture (for pST assay). The capsule site was also assessed for immune responses (e.g. lymphocyte infiltration).

The results of measurements of mean (3 hr, 30 min interval) plasma pST concentration of pigs before and 7 days after receiving pST capsules (secreting between 5 and 1000 ng/ml) are shown in Figure 8. As can be seen from Figure 8, it is apparent that plasma pST is reduced in pigs following 1 week exposure to immunoneutral pST (5 - 100 ng/ml) secreting capsules.

The variability between and within individual plasma pST concentrations appeared to be more apparent during the control serial sampling period. This phenomenon is reflected in the Standard Errors about the mean observed concentrations. Further, the stable baseline and pST pulse intervals (normally 3 - 4 hrs) were not recognised by computer programs designed to identify hormone pulses. However, stable baselines and distinct pST pulses were observed in animals 1 week post pST casule administration (Figure 9).

The Rate of Gain (ROG) shown by the animals appeared to be responsive to pST capsule secretion in a dose dependent manner (Figure 10). A secretion rate of 30 ng/ml (i.e. 3 capsules secreting 10 ng/ml each) appears to be the minimum dose required to observe growth rate increases. The majority of evc's remained patent for 21 days at which time, the animals were euthanased with barbituate for carcass analysis. Analysis of carcass back fat (P2 without skin) measurements further indicate that 30 ng.ml is the minimum dose to observe energy repartitioning within 21 days of pST capsule administration (Figure 10).

Throughout the experiment there were no indications of adverse reactions, reduction in weight gain or adverse immune responses, including those animals that received 100 capsules.

Example 5: Pilot experiment (3) involving implantation of pST-L6IXS in pigs

Following example 4, investigations were conducted to assess the effect of the administering optimal pST secretion rates/capsule numbers to pigs at varying times prior to slaughter (i.e. 2, 4 and 6 weeks prior to slaughter) on back fat. 8 pigs were used for each treatment as well as 8 control (i.e. no pST capsules).

The results of the Rate of Gain measurements are provided in Figure 11.

Back fat measurements were obtained following whole carcass chilling (24 hours @ 4°C) (Figure 12). P2 measurements were recorded at the 12th rib 65mm from the centre of the spinal column. Pigs exposed to capsules secreting pST for 2, 4 and 6 weeks were observed to have significantly reduced back fat. This effect in the 2 and 6 week period is approximately a 46% reduction in back fat. The animals exposed to pST IGT capsules for 4 weeks were more variable in their back fat responses, which may relate to a possible failure to recover all the capsules from a number of these animals.

Loin muscle area in pigs exposed to secreting capsules was only significantly increased (i.e. 22 %) following 6 weeks exposure to pST IGT capsules (Figure 13).

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

References:

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